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Identification of Thormählen-Positive Compound "B" in Urine of Patients with Malignant Melanoma

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Summary. The identification of the entire structure of the Thormählen-positive compound B from melanotic urine is described. The compound was separated from other Thormählen-positive compounds using DEAE-cellulose column chromatography. On the basis of differential enzymic hydrolysis followed by gas chromatography-mass spectrometry analysis and comparison with synthetically prepared compounds it is possible to conclude that the Thormählen-positive compound B is a mixture of O-sulphate 5-hydroxy-6-methoxyindole and 6-hydroxy-5-methoxyindole with predominance of the latter.

Key words: Melanoma – Melanotic urine – Indoles – Sulphate conjugates

Introduction

The Thormählen reaction (Thormählen 1887) has proven successful in recent years as a criterion for the classification of excreted phenolic and indolic compounds in melanotic urine. This simple reaction (see 'Materials and Methods') makes it possible to detect indolic compounds which in Leonhardi's conception possess an unsubstituted pyrrole ring in the indole nucleus (Leonhardi 1953). Using paper chromatography, Leonhardi was the first to describe three Thormählen-positive (TP) substances – denominated A, B and C – in the urine of melanoma patients (Leonhardi 1954).

In 1981, we reported the identification of TP compound A and the associated AX as glucuronides of 5-hydroxy-6-methoxyindole (5H6MI) and 6-hydroxy-5-methoxyindole (6H5MI), respectively (Pavel et al. 1981a).

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This paper describes the identification of the structure of TP substance B partially isolated from melanotic urine using DEAE-cellulose column chromatography. Differential enzymatic hydrolysis, gas chromatography-mass spectrometry (GC-MS) analysis and comparison with synthetically prepared compounds contributed to resolving the entire structure of the TP compound.

Materials and Methods

Chemicals

3,4-Dihydroxyphenylethylamine (dopamine) and arylsulphatase [EC 3.1.6.1] (from *Aerobacter aerogenes*) were purchased from Sigma (St. Louis, MO, USA); β-glucuronidase [EC 3.2.1.31] (from *Escherichia coli*) was from Boehringer (Mannheim, FRG); pentafluoropropionic anhydride (PFPA) and 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) were purchased from Pierce (Rockford, IL, USA): DEAE-cellulose was obtained from Fluka AG (Buchs, Switzerland), sulphur trioxide trimethylamine complex from Aldrich-Europe (Beerse, Belgium), and *Helix pomatia* juice was from l'Industrie Biologique Française (Gennevilliers, France). All other chemicals were from Merck (Darmstadt, FRG).

Isolation of TP Compound B from Melanotic Urine

Melanotic urine with a high content of TP compounds was used. Of the urine 10 ml was applied to a 1 × 50 cm column with DEAE-cellulose. The column was washed with 100 ml water and the elution carried out with a non-linear gradient of ammonium acetate (300 ml 0.4 mol × l^{-1}, 300 ml 0.8 mol × l^{-1}). The content of TP compounds in 5 ml fractions was tested using Thormählen reaction and thin-layer chromatography (TLC). The fractions containing compound B (viz. No. 28–31) were pooled and freeze-dried. The residue was dissolved in 1 ml water and used for a structural study.

Enzymatic Hydrolysis, Extraction and Derivatization

The solution containing the partially isolated TP substance B was divided into four tubes each containing 200 µl. To each tube 1 ml water was added followed by 500 µl 2.5 mol × l^{-1} sodium acetate buffer (pH 6.2). The same buffer with pH 7.0 was used for hydrolysis with arylsulphatase. 100 µl *Helix pomatia* preparation, 20 µl β-glucuronidase and 200 µl arylsulphatase were added to the appropriate tubes which were then placed in a vibrating waterbath at 37°C.

The tube without enzyme served as a control. After 120 min incubation, the tube were extracted with 2 × 4 ml diethyl ether and the pooled extracts dried over anhydrous sodium sulphate and evaporated to dryness. Derivatization was carried out with 100 µl PFPA and heating in a heating block at 60° C for 10 min. The rest of the PFPA was then evaporated and the residue dissolved in 20 µl of freshly prepared ethyl acetate containing 5% PFPA. The solutions were used for GC-MS analysis.

Synthesis of Sulphoconjugates of 5H6MI and 6H5MI

5H6MI and 6H5MI were prepared by methylation of 5,6-dihydroxyindole (5,6DHI) as described elsewhere (Pavel and Muskiet 1983b). The methylation of about 20 mg 5,6DHI was checked by gas chromatography and terminated when no 5,6DHI could be detected. The ethyl acetate was then evaporated, the residue dissolved in 500 µl dry pyridine, and 200 mg solid sulphur trioxide trimethylamine complex was added. The mixture was then kept at room temperature overnight. The reaction product was dissolved in 5 ml water, centrifuged, and the supernatant applied to the column of DEAE-cellulose. Washing, elution and collection of fractions were performed as described above. Fractions containing sulphoconjugates of 5H6MI and 6H5MI were freeze-dried and examined by TLC.

Thormählen Reaction

Of the sample 1 ml was mixed with 250 µl freshly prepared 1% sodium nitroprusside and 500 µl 10% KOH. After approximately 2 min the solution was acidified with 50 µl concentrated acetic acid. If necessary, smaller parts had been used in the same ratios. Absorbance was measured at 620 nm.

TLC

TLC was carried out on cellulose plates (PSC-Fertigplatten Cellulose 20 × 20 cm, layer thickness 0.5 mm; Merck, Darmstadt, FRG) in n-butanol:pyridine:water (1:1:1) solvent system. The spots were made visible by the Thormählen (Crawhall et al. 1967) and Ehrlich (Jepson 1960) reactions.

GC and GC-MS

GC was performed on a Packard-Becker Model 417 gas chromatograph as described elsewhere (Pavel and Muskiet 1983b).

GC-MS analyses were performed with a Varian 3700 gas chromatograph interfaced to a Varian MAT 44 S mass spectrometer with an open-split coupling. Data were recorded on a Finnigan MAT SS 200 data system. The gas chromatograph contained a 15 m × 0.25 mm (i.d.) glass capillary column coated with SE-54 (Franzen Analyzen-Technik, Bremen, FRG).

Injector temperature was set at 240° C, interface temperature at 250° C, and source temperature at 200° C. The oven temperature was programmed from 120 to 230° C at increases of 5° C/min.

Results

The elution profile of the TP compounds from melanotic urine is show in the upper part of Fig. 1. As can be seen from this picture, DEAE-cellulose chromatography provided the base-line separation of all three main TP substances. TLC on cellulose plates then made it possible to identify compounds as A, B and C according to their chromatographic mobility and colour reactions (Fig. 1, lower part). Under the considered

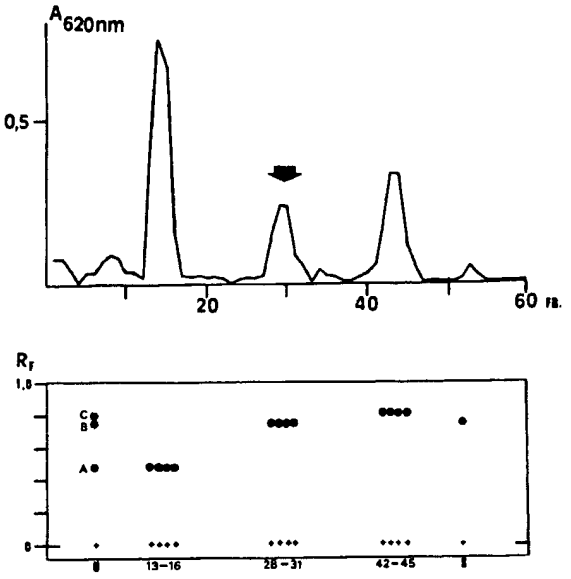

Fig. 1. *Upper:* A profile of TP compounds eluted from DEAE-cellulose column. The *arrow* shows the position of fractions containing TP substance B. Thormählen reaction – see Material and Methods. *Lower:* A scheme of TLC of eluted fractions with TP substances A (No. 13–16), B (No. 28–31), and C (No. 42–45). For comparison: *U* untreated melanotic urine; *S* synthetically prepared mixture of 5-methoxy-6-sulphonyloxyindole and 6-methoxy-5-sulphonyloxyindole. Detection – Thormählen reaction

condition of consistent molar absorption coefficients of urinary TP compounds one could estimate that TP substance B formed approximately 19% of the total separated TP compounds (A, 55%; C, 26%).

TLC detection with Ehrlich reagent showed, however, that fractions containing substance B (No. 28–31) were contaminated by (Thormählen negative) indoxyl sulphate, a physiological constituent of normal urine, which gave a typical orange colour after spraying. This compound, however, did not interfere with further procedures, because GC-MS analysis provided a good separation of impurities (GC) and very specific detection of separated compounds (MS).

A mixture of 5-methoxy-6-sulphonyloxyindole and 6-methoxy-5-sulphonyloxyindole was prepared synthetically (see 'Materials and Methods'). The subsequent TLC comparison showed a close similarity of synthetized compounds with that partially purified TP compound B. Differential enzymic hydrolysis confirmed our presumption that TP substance B could only be hydrolyzed with arylsulphatase and *Helix pomatia* juice which also contains arylsulphatase activity. The hydrolysis was monitored by mass chromatography (Fig. 2) and the structures of those liberated 5H6MI and 6H5MI were confirmed by their mass spectra (Fig. 3) which can be interpreted as follows:

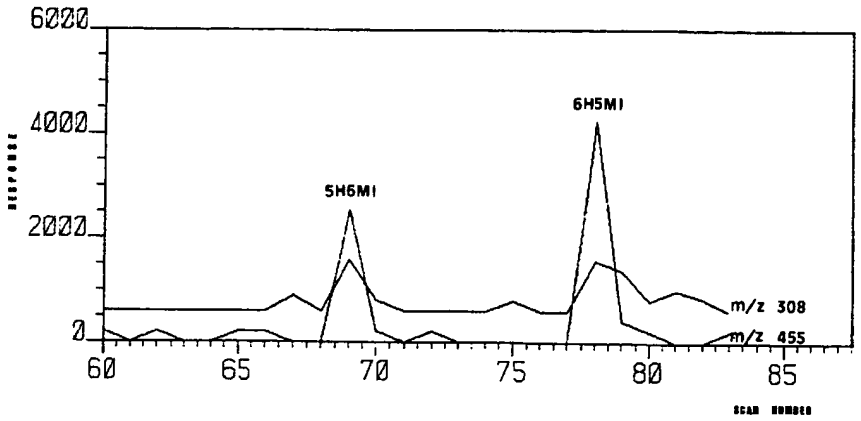

Fig. 2. A mass chromatogram indicating the presence of the PFP derivatives of 5H6MI and 6H5MI in the partially isolated TP compound B after hydrolysis by arylsulphatase, extraction and derivatization

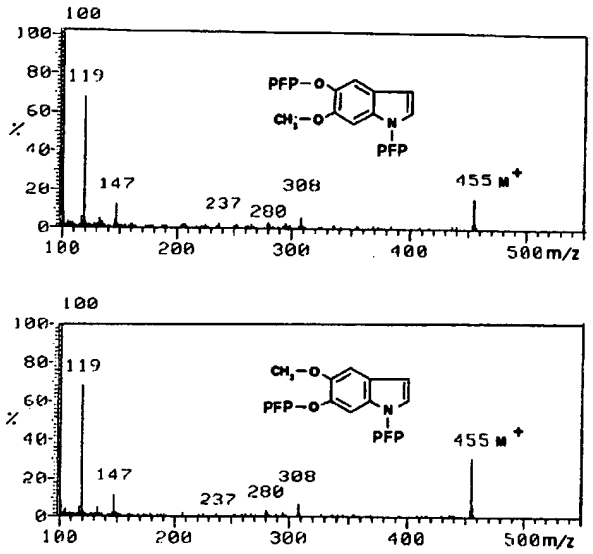

Fig. 3. Mass spectra of the PFP derivatives of 5H6MI (*upper*) and 6H5MI (*lower*) from TP substance B (scan number 69 and 78, respectively)

m/z 455 = $[M]^+$; m/z 308 = $[M-PFP]^+$; m/z 280 = $[M-(PFP-N=CH_2)]^+$; m/z 237 = $[M-(CH_3O + PFP-N=CH_2)]^+$; m/z 147 = PFP; m/z 119 = pentafluoroethyl. The estimation of the peak areas of monitored methoxyhydroxyindoles provided the surprising information about the TP compound B being predominantly composed from 6H5MI-sulphate.

Discussion

The presence of 5-methoxy-6-sulphonyloxyindole and 6-methoxy-5-sulphonyloxyindole in melanotic urine is in agreement with our recently formulated conception of melanogenesis in malignant melanoma (Pavel et al. 1983), and thus the biosynthesis of the TP compound B can be seen as follows: A part of 5,6DHI which had not been used for melanin formation is efficiently methylated by catechol-O-methyltransferase in melanocytes. The methylated products (5H6MI and 6H5MI) are released from the cells and transported in blood to be conjugated with glucuronate and sulphate in the liver and excreted from the body. If a sufficient amount of these conjugates is produced, it is possible to detect TP spots on paper or thin-layer chromatograms of melanotic urine.

Experience has shown that from all the three mentioned TP compounds, A has always been the most intense spot on urinary chromatograms. It is therefore possible to assume that methoxyhydroxyindoles are preferentially O-conjugated with glucuronate. This is especially the case of 5H6MI which has been shown to be the compound with the highest excretion from all the indolic compounds in generalized malignant melanoma (Pavel et al. 1981b; Pavel and Muskiet 1983a). Conjugation with sulphate, in the second place, leads to formation of O-sulphated 5H6MI and 6H5MI. Our results show the preferential formation of 5-methoxy-6-sulphonyloxyindole.

Of the main TP compound, the structure of C remains unknown. However, our recent investigations provide hope that the entire structure of these compounds will be resolved in the very near future.

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